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Circulating CD34$^+$/KDR$^+$ endothelial progenitor cells are reduced in chronic heart failure patients as a function of Type D personality

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ABSTRACT

The aim of the present study was to assess whether EPC (endothelial progenitor cell) number/function might be an explanatory factor for the observed relationship between Type D personality (a joint tendency towards negative affectivity and social inhibition) and poor cardiovascular prognosis. We also assessed whether the effect of a single exercise bout on EPC number/function was affected by Type D personality. A total of 35 sedentary men with CHF (chronic heart failure; left ventricular ejection fraction $\leqslant 45\%$) underwent CPET (cardiopulmonary exercise testing) and personality assessment with the 14-item Type D scale. CD34$^+$/KDR (kinase insert domain-containing receptor)$^+$ cells were quantified by flow cytometry before and immediately after CPET. Migration of early EPC towards VEGF (vascular endothelial growth factor) and SDF-1α (stromal-cell-derived factor-1α) was investigated. Type D ($n = 10$) and non-Type D ($n = 25$) patients were comparable with regards to demographics, disease severity and Framingham risk factor score. Circulating EPC numbers were reduced by 54% in Type D compared with non-Type D patients (0.084 ± 0.055 and $0.183 \pm 0.029\%$ of lymphocytes respectively; $P = 0.006$). Exercise led to a 60% increase in EPC in Type D patients, whereas the EPC number remained unchanged in the non-Type D group ($P = 0.049$). Baseline migratory capacity was related to disease severity, but was not different between Type D and non-Type D patients. Exercise induced a highly significant enhancement of migratory capacity in both groups. In conclusion, reduced EPC numbers might explain the impaired cardiovascular outcome in Type D patients. The larger increase in circulating EPCs observed in these patients suggests that acute exercise elicits a more pronounced stimulus for endothelial repair.

Key words: cardiopulmonary exercise test, chronic heart failure, endothelial progenitor cell, endothelium, migration, Type D personality.

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II type 1 receptor blocker; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; CHF, chronic heart failure; CPET, cardiopulmonary exercise test; DS14, 14-item Type D scale; EPC, endothelial progenitor cell; FMD, flow-mediated dilation; IMT, intima media thickness; KDR, kinase insert domain-containing receptor; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; NT-proBNP, N-terminal pro-brain natriuretic peptide; TNF-α, tumour necrosis factor-α; $\dot{V}CO_2$, carbon dioxide production; $\dot{V}E$, expired minute ventilation; $\dot{V}O_2$, oxygen consumption; $\dot{V}O_{2peak}$, peak $\dot{V}O_2$.

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INTRODUCTION

Type D personality, a joint tendency toward negative affectivity and social inhibition, is associated with poor prognosis in cardiovascular patients [1]. The relationship between emotional distress and cardiovascular disease has been linked to an unhealthy lifestyle, but also to more physiological processes, such as disturbed autonomic control [2], impaired platelet function [3] and a pro-inflammatory environment [4]. Recently, decreased numbers of EPCs (endothelial progenitor cells) have been suggested as another possible mediator of cardiovascular disorders in depressed patients [5]. In line with this, psychosocial risk factors are related to levels of circulating progenitor cells, independent of classical cardiovascular risk factors [6].

Accumulating evidence suggests that the quantity and function of circulating EPCs play an important role in preserving endothelial integrity. These cells are released from the bone marrow in response to ischaemia or endothelial injury with the aim of repairing damaged regions either by producing angiogenic cytokines [7] or by differentiating into endothelial cells [8]. Besides re-endothelialization, EPCs play a role in post-ischaemic neoangiogenesis [9]. The therapeutic use of EPCs and bone marrow mononuclear cells after vascular injury appears promising [10,11], but safety issues need to be addressed further [12].

In patients at cardiovascular risk [13], in those with CAD (coronary artery disease) [14] and in CHF (chronic heart failure) patients [15], the number of circulating EPCs is reduced. In addition to this quantitative deficit, a subnormal function of EPCs correlates with impaired endothelial function [16]. Diversity in terms of EPC definition and quantification methods has complicated cross-study comparisons [17], sometimes leading to paradoxical findings, such as increased numbers of EPCs in patients with higher cardiovascular risk [18].

Psychological distress promotes immune dysregulation [19], which in turn might mediate bone-marrow suppression or cause depletion of EPCs in the peripheral circulation. We have demonstrated previously that Type D personality in CHF patients is independently associated with increased circulating levels of TNF-α (tumour necrosis factor-α) and its soluble receptors [20], which have been shown to predict poor outcome in CHF [21,22].

Physical training has proved to be of clear benefit in cardiovascular patients. Besides the central haemodynamic effect, exercise tolerance is improved by partially restoring vascular reactivity. Shear-stress-induced up-regulation of eNOS (endothelial NO synthase) and a decrease in oxidative stress play a central regulatory function. Moreover, recent studies have demonstrated that regular physical exercise mobilizes EPCs in CAD and CHF patients [23], which might explain the observed benefit on endothelial function. An acute exercise bout in sedentary patients, however, poses a threat to endothelial integrity by inducing vascular oxidative stress and a transient prothrombotic stimulus.

In the present study, we investigated the relationship between Type D personality and EPC numbers and their function in CHF patients before and after endothelial stress elicited by a symptom-limited CPET (cardiopulmonary exercise test).

MATERIALS AND METHODS

Patients

A total of 35 consecutive sedentary male CHF patients with an LVEF (left ventricular ejection fraction) $\leqslant 45\,\%$, followed at the outpatient heart failure clinic of the Department of Cardiology, were recruited for the present study. Patients were stable with regard to symptoms and therapy for at least 1 month, and were on standard medical treatment consisting of ACEIs (angiotensin-converting enzyme inhibitors) or ARBs (angiotensin II type 1 receptor blockers), β-blockers, diuretics and spironolactone as indicated. Patients with active infection or cancer and patients on anti-inflammatory drugs were excluded.

The study complies with the Declaration of Helsinki, was approved by the local ethics committee and written informed consent was obtained from all patients.

Study design

Complete clinical assessment, including resting BP (blood pressure) measurement and NYHA (New York Heart Association) classification, was performed at the time of enrolment. The total burden of cardiovascular risk factors was calculated using the Framingham risk factor score [24]. LVEF was determined using echocardiography (Simpson's rule). Fasting blood samples were collected in order to measure complete blood cell count, and creatinine, glucose and lipid levels. Subjects performed a symptom-limited CPET on a graded bicycle ergometer. Immediately before and 10 min after peak exercise, venous blood samples were drawn from an antecubital vein and collected in acid citrate dextrose tubes. Samples were processed immediately or stored at $4\,^{\circ}C$ and analysed within 4 h.

Clinical data and Type D personality

All 35 patients completed a DS14 (14-item Type D scale). DS14 comprises a seven item subscale measuring negative affectivity and a seven item subscale measuring social inhibition. A cut-off of $\geqslant 10$ on both DS14 subscales was used to classify patients as Type D personality [25].

CPET

To obtain an optimal duration of CPET between 8 and 10 min, two ramp protocols were used: patients started either with 20 or 40 W, with an incremental load of 10 compared with 20 W every minute respectively. A 12-lead ECG and heart rate were recorded continuously, whereas automatic cuff BP was measured every 2 min and at peak exercise. Breath-by-breath gas exchange measurements allowed online determination of $\dot{V}_E$ (expired minute ventilation), $\dot{V}O_2$ (oxygen consumption) and $\dot{V}CO_2$ (carbon dioxide production) every 15 s. $\dot{V}O_{2peak}$ (peak $\dot{V}O_2$) was determined as the highest attained $\dot{V}O_2$ during the final 30 s of exercise and was also expressed as a percentage of the predicted value. Online analysis of $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ curves permitted the researchers to encourage patients to exercise up to exhaustion.

Assessment of endothelium-dependent vasodilation and IMT (intima media thickness)

Endothelium-dependent vasodilation was assessed in supine position by ultrasound of the right brachial artery (AU5 ultrasound system; Esaote Biomedica). End-diastolic diameters were evaluated at rest and during reactive hyperaemia after inflating and deflating a forearm BP cuff (200 mmHg or at least 50 mmHg above peak systolic BP for 4 min). Post-occlusion measurements were taken every 30 s and over the following 240 s. FMD (flow-mediated dilation) was determined as the percentage change in diameter between baseline and maximal post-occlusion values.

IMT was measured with high-resolution ultrasound at the posterior wall of the common carotid artery using an automated edge-tracking method.

Quantification of circulating CD34$^+$/KDR (kinase insert domain-containing receptor)$^+$ cells

CD34 and KDR, commonly used membrane markers to define EPCs, were detected on cells by flow cytometry [26]. In brief, 200 μl of peripheral whole blood was pre-treated with an FcR (Fc-receptor)-blocking reagent (Miltenyi) to prevent non-specific binding and were incubated with a PE (phycoerythrin)-conjugated anti-KDR antibody (R&D Systems) and a PeCy7-conjugated anti-CD34 antibody (BD Biosciences). After red blood cell lysis with ammonium chloride (Stem Cell Technologies), a total of 10^6 events was recorded on a FACSCantoII flow cytometer (BD Biosciences). Time was entered as a parameter to facilitate the identification and removal of event bursts and minor clogs. Before analysis, the flow cytometer was thoroughly cleaned to remove residual cells. Fluorochrome- and isotype-matched controls, as well as unstained cell samples, were measured and processed as negative controls to set the appropriate regions. The numbers of CD34$^+$/KDR$^+$ cells were analysed in the lymphocyte region using FACSDiVa software and are expressed as a percentage of lymphocytes.

Migratory activity of early EPCs

PBMCs (peripheral blood mononuclear cells) were isolated by density gradient centrifugation with Lymphosep (MP Biomedicals) and were cultured on fibronectin-coated 24-well dishes in EGM-2 MV (Clontechs) supplemented with 20 % (v/v) fetal calf serum. On day 7, cells were characterized as early EPCs by the uptake of acetylated LDL [DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)-acLDL; Molecular Probes] and binding of FITC-conjugated lectin UEA-1 (*Ulex europaeus* agglutinin-1; Sigma). Migratory activity of EPCs towards VEGF (vascular endothelial growth factor; 50 ng/ml; R&D Systems) and SDF-1α (stromal-cell-derived factor-1 α; 100 ng/ml; R&D Systems) was determined using 5 μm transwells (Corning Costar). After 4 h, viable EPCs that had migrated to the lower chamber were stained with Trypan Blue and counted manually using a haemocytometer. EPCs counted in the negative control transwell (no addition of chemoattractants) were subtracted from this number. Migrated EPCs were expressed as a percentage of the total cell number added to the transwell (positive control).

Statistical analysis

Continuous data are expressed as means $\pm$ S.E.M. Normality of data was assessed using a one-sample Kolmogorov–Smirnov test. Independent-sample Student t and χ^2 tests were used as appropriate for baseline measurements. Pre- and post-exercise data were compared in the two groups using paired sampled Student's t tests with Levene's test for equality of variances. Calculating the difference in mean EPC levels between Type D and non-Type D patients, and dividing it by the S.D. of the total group at baseline, estimated an effect size of Type D personality. An effect size of 0.50 reflects a moderate effect, whereas 0.80 is a large effect [27]. In order to estimate the magnitude of the Type D effect on EPC numbers, we compared its effect size with those of older age and standard cardiovascular risk factors. A median split was used to dichotomize EPC levels in order to stratify patients into high- and low-risk groups respectively. Multiple logistic regression (method = enter) was used to determine the risk associated with Type D personality after controlling for age, BMI (body mass index) and LVEF.

All analyses were performed using SPSS 16.0 for Windows. A two-tailed P value of < 0.05 was considered statistically significant.

Table 1 Demographic and clinical characteristics as a function of Type D personality

Values are means ± S.E.M., or numbers (%).

Characteristic	Non-Type D ($n = 25$)	Type D ($n = 10$)	P value
Age (years)	63.4 ± 2.6	56.3 ± 3.0	0.1
BMI (kg/m^2)	27.0 ± 0.7	25.5 ± 1.6	0.4
Framingham risk factor score	4.5 ± 0.6	5.2 ± 1.0	0.5
Diabetes mellitus (n)	2 (8%)	1 (10%)	0.7
Creatinine clearance rate (ml/min)	62.7 ± 6.7	69.4 ± 6.4	0.6
NYHA class I/II/III (n)	5/11/9	0/8/2	0.1
Ischaemic aetiology (n)	19 (76%)	6 (60%)	0.3
LVEF (%)	27.6 ± 1.9	26.5 ± 2.1	0.7
NT-proBNP (pg/dl)	1074 ± 199	850 ± 497	0.6
IMT (μm)	650.3 ± 23.0	621.2 ± 40.0	0.5
FMD (%)	5.2 ± 0.3	6.2 ± 1	0.2
$\dot{V}o_{2peak}$ (ml·kg^{-1} of body weight·min^{-1})	20.5 ± 2.0	23.7 ± 2.4	0.4
Percentage of predicted $\dot{V}o_{2peak}$	81.9 ± 4.6	83.0 ± 8.1	0.9
$\dot{V}_E/\dot{V}_{CO_2}$ slope	28.8 ± 1.3	25.5 ± 2.0	0.2
Medication (n)			
ACEIs	23 (92%)	9 (90%)	0.9
ARBs	2 (8%)	1 (10%)	0.7
β-Blockers	22 (88%)	10 (100%)	0.5
Diuretics	20 (80%)	8 (80%)	1.0
Spironolactone	11 (44%)	3 (30%)	0.5
Statins	19 (76%)	5 (50%)	0.3

RESULTS

Patient characteristics according to Type D classification

According to DS14, ten out of the 35 CHF patients enrolled were classified as Type D. Demographic characteristics, NYHA classification, LVEF, FMD, IMT, disease aetiology, cardiovascular risk factors and medication were comparable in Type D and non-Type D patients (Table 1). None of the patients received any other medication with known effects on EPC number and function [i.e. PPAR-γ (peroxisome-proliferator-activated receptor-γ) agonists, calcium antagonists, organic nitrates or PDE-5 (phosphodiesterase-5) inhibitors].

Baseline number of circulating CD34$^+$/KDR$^+$ cells and function of early EPCs

As shown in Figure 1, circulating EPC numbers were significantly reduced ($P = 0.006$) by 54% in Type D patients (0.084 ± 0.055% of lymphocytes) compared with non-Type D patients (0.183 ± 0.029% of lymphocytes).

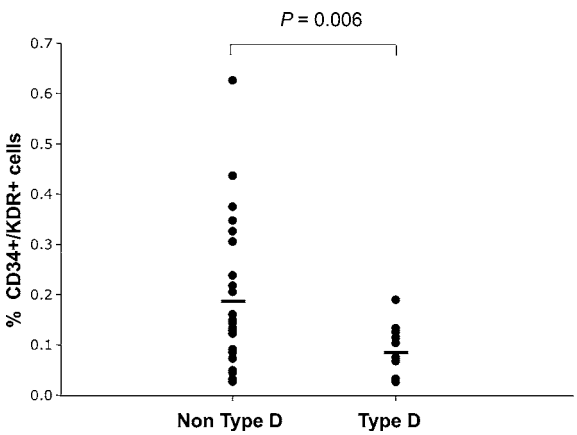

Figure 1 Comparison of circulating EPC numbers in CHF patients with Type D ($n = 10$) and non-Type D ($n = 25$) personality

EPCs (CD34$^+$/KDR$^+$ cells) are expressed as a percentage of total lymphocytes.

Figure 2 shows the importance of Type D personality as a determinant of EPC number. An effect size of 0.8 indicated that Type D personality had a large effect on EPC numbers. In comparison, advanced age (> 65 years) (effect size = 0.46) and unfavourable standard cardiovascular risk profile (effect size = 0.17) had small effects (Figure 2). Hence the effect of Type D personality was larger than that of these well-known determinants of EPC numbers. Apart from Type D personality, age, BMI and LVEF were included in a multiple logistic regression model in order to control for factors known to influence EPC numbers [15,28,29]. This model retained Type D personality as an independent predictor of low EPC

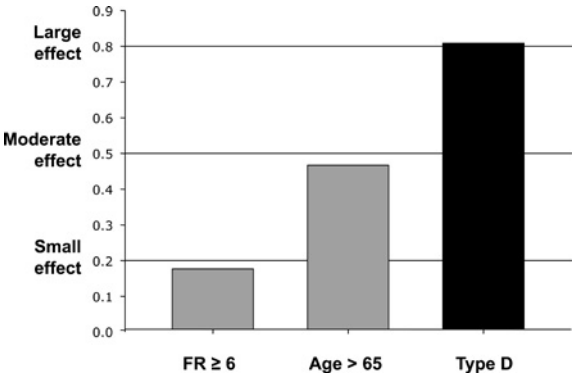

Figure 2 Effect size of advanced age, unfavourable cardiovascular risk profile and Type D personality as determinants of the number of circulating EPCs

An effect size of 0.50 reflects a moderate effect and 0.80 a large effect on the number of circulating EPCs. FR, Framingham risk score $\geqslant 6$ (unfavourable cardiovascular risk profile); Age > 65 (years), advanced age.

levels [odds ratio, 8.2 (95 % confidence interval, 1.2–57.4); $P = 0.034$].

The number of circulating EPCs was not related to determinants of disease severity such as poor LVEF and reduced $\dot{V}O_{2peak}$ (all $P > 0.05$).

Migratory capacity correlated with the percentage of the predicted $\dot{V}O_{2peak}$ ($r = 0.334$, $P = 0.053$) and inversely with NT-proBNP (N-terminal pro-brain natriuretic peptide) levels ($r = -0.409$, $P = 0.031$). However, the migratory activity of early EPCs was not different in Type D compared with non-Type D patients (34.9 ± 3.4 and 37.1 ± 5.4 % respectively; $P = 0.73$).

Effect of exercise on EPC number and function

Changes in the number of circulating EPCs and migratory activity of early EPCs following CPET are shown in Figure 3. A single exercise bout led to a 60 % increase in EPCs in Type D patients, whereas the EPC number remained unchanged in the non-Type D group ($P = 0.049$; Figure 3A). Migratory capacity of early EPCs improved significantly after exercise in both Type D and non-Type D patients (Figure 3B). This increase was comparable in the two groups ($P = 0.97$).

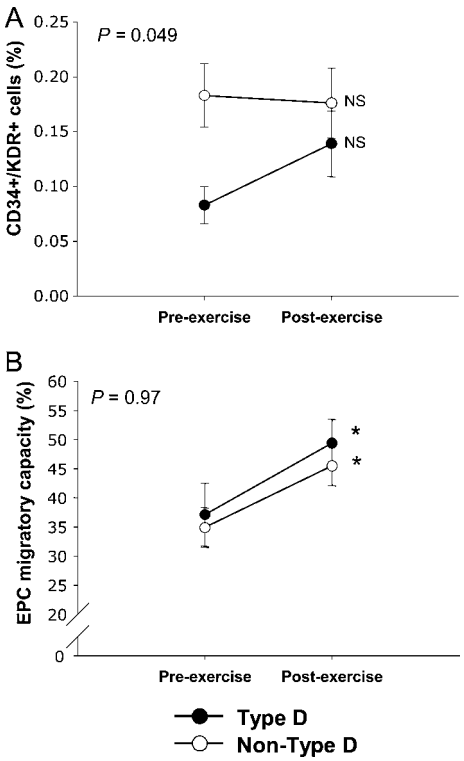

Figure 3 EPC numbers (A) and migratory capacity (B) at baseline and after a single maximal exercise bout in CHF patients with Type D ($n = 10$) and non-Type D ($n = 25$) personality

(A) EPCs ($CD34^+/KDR^+$ cells) are expressed as a percentage of total lymphocytes. The change in EPCs following exercise was significantly different in Type D patients compared with non-Type D patients. (B) EPC migratory capacity is increased following exercise in both Type D and non-Type D patients. *P* values are given for differences between Type D and non-Type D patients. $^*P < 0.05$ compared with baseline; NS, not significant compared with baseline.

DISCUSSION

Depletion and altered function of EPCs have been demonstrated in CHF, which is characterized by endothelial dysfunction. In cardiac patients, advanced age and an unfavorable cardiovascular risk profile [14] are well-known determinants of reduced circulating EPC numbers. The results of the present study are the first to suggest that Type D personality may also be associated with a significantly impaired release and or/survival of circulating EPCs in CHF patients.

The potential importance of Type D personality was demonstrated by its large effect size compared with that of standard clinical factors affecting EPC numbers. Individuals with Type D personality tend to experience ongoing situations in their lives as stressful, which, in turn, may elicit negative emotional reactions and physiological hyper-reactivity every time a potentially 'threatening' situation is encountered [30]. Previous studies have demonstrated that Type D personality is independently associated with increased concentrations of the pro-inflammatory cytokine TNF-α and its soluble receptors [20], increased levels of the stress hormone cortisol [31] and a disrupted autonomic balance [32]. These physiological stress mechanisms may help to clarify the link between Type D personality and reduced EPC numbers in the present study.

Dome et al. [5] recently showed an imbalance of pro- and anti-inflammatory cytokines in patients with depression, which was associated with reduced EPC numbers. In the study by Valgimigli et al. [15],

lower numbers of circulating EPCs in severe CHF were linked to increased serum concentrations of TNF-α. *In vitro* experiments have demonstrated that TNF-α causes growth suppression of bone marrow $CD34^+$ haemopoietic stem cells [33] and induces apoptosis of peripheral circulating EPCs [34]. In line with this, it is conceivable that the increased TNF-α activity observed in Type D personality [35] induces the suppression of bone marrow haemopoietic stem cells, thereby limiting the number of circulating progenitor cells.

Activation of the HPA (hypothalamic–pituitary–adrenal) axis in Type D personality and a subsequent increased production of cortisol and catecholamines [32,36] could provide a second plausible mechanism. Both endogenously produced, as well as synthetic, steroids induce substantial losses of lymphoid precursor cells in the bone marrow via apoptotic pathways [37,38]. Moreover, hydrocortisone hampers the differentiation of human $CD34^+$ cells towards lymphocytes in *in vitro* experiments [39].

Thirdly, it is conceivable that the disrupted autonomic balance in Type D personality [2] partially accounts for the lower EPC counts. The bone marrow is supplied by autonomic sympathetic efferent innervation [40], which plays a role in the proliferation and release of haemopoietic cells from the bone marrow [41].

The maintenance of an intact endothelial monolayer, either by adjacent mature endothelial cells or by EPCs, prevents the development of atherosclerosis. EPCs also affect disease progression and determine prognosis independently of known cardiovascular risk factors [14]. To a large extent, endothelium-dependent vasodilation determines the functional capacity of patients. Measures taken to ameliorate endothelial vasoreactivity, such as physical training, partially restore endothelial dysfunction [22], and EPCs have been suggested as key regulators. An acute exercise bout, however, poses a threat to endothelial integrity by inducing vascular oxidative stress and a transient prothrombotic stimulus. It has been shown in healthy subjects that circulating EPC numbers increase following a maximal exercise bout [42], thereby supporting the conjecture that the generation of ROS (reactive oxygen species) may trigger EPC release. The findings of the present study demonstrate that the exercise-induced stimulus for endothelial restoration is larger in Type D patients than in non-Type D patients. The more pronounced release of EPCs might be considered a mechanism of defence in order to compensate for the observed chronic deficit in circulating EPC numbers at rest.

Although the number of circulating EPCs was lower in Type D patients, their migratory capacity was not. However, recent evidence suggests that $CD34^+/DKR^+$ cells and so-called 'early EPCs', cultivated *in vitro*, are in fact different EPC subpopulations, characterized by other antigenic cell-surface markers and different angiogenic properties [43].

The present findings should be interpreted with caution, as the number of patients investigated was limited. Nevertheless, the effect size for Type D personality was remarkably high in comparison with known determinants of circulating EPC numbers. The absence of a relationship between EPC numbers and disease severity in this small group of patients might be explained by the fact that the group was rather homogenous. In addition, the main purpose of the present study was to try to identify disease-modifying mechanisms that might help to explain why Type D patients are prone to repeated cardiovascular events and poor prognosis.

Several studies have shown that Type D personality predicts poor prognosis in cardiac patients. The exact biological pathways by which Type D personality predisposes to cardiovascular disease still awaits further clarification. The findings of the present study are the first to suggest that Type D personality may contribute to the pathophysiology of cardiovascular disease by mediating the number of circulating EPCs. Future studies should be designed to investigate possible underlying mechanisms using both *in vivo* and *in vitro* experiments.

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